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<b>14. ABSTRACT</b> Cell-Extracellularmatrix (ECM) adhesions structurally alter the cells aswell as provide signals critical for the cell differentiation and tissue-specific gene transcription. We show edthat the ECM induce global deacetylation of his tones H3 and H4 in human breast epithelial cells, and that this deacetylation correlated with a global reduction in gene expression. To determine how chromatin structure regulates differentiated gene expression in 3D culture, we used mammary-specific case in genes as models. We identified that transcription factors Stat5 and C/EBP beta were regulated by ECM. Chromatinimmunoprecipitation(ChIP) assays demonstrated that levels of acetylated histones as well as binding of Stat5 and C/EBP betain the case inpromoters increased in response to ECM and prolactin treatment. However, trichostatin A(TSA)-induced histoneacetylation failed to activate•-case in expression, suggesting that histoneacetylationis not sufficient for the gene transcription. Introduction of adominantnegative Brg1 significantly reduced both beta-andgamma-casein expression, indicating that ATP-depend entchromatinremodeling is required for transcription of these two milk proteinges. Thus, ECM-and prolactin-regulated transcription of the mammary-specific case in genes requires the concertedaction of histoneacetylation,ATP-depend entchromatinre modeling, andctivation of transcription factors.						
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## Introduction:

In specific aim I, we proposed to determine if ECM-mediated transcription down regulation during HMEC differentiation corresponds with a change in levels of histone acetylation in the specific regulatory DNA region using EGFR gene as a model. However, the quantitative RT-PCR showed that there was no significant difference in EGFR mRNA levels between cells cultured on plastic and cultured in 3D, suggesting that EGFR gene is not a good model to study the transcriptional regulation induced by extracellular matrix (ECM) during the differentiation of mammary gland epithelial cells. We showed that global histone acetylation levels decreased in three dimensional (3D) cultures. Microarray analysis demonstrated that this deacetylation correlated with a global reduction in gene expression. These results addressed the question in specific aim I at the whole genome level. Based on our microarray data, we used p21 gene as a model to study the histone acetylation in a specific chromatin region. Chromatin immunoprecipitation data showed that ECM induced a dramatic reduction of histone H4 acetylation associated with the p21 promoter, which is consistent with down regulation of p21 mRNA levels in 3D cultures.

In specific aim II, we proposed to identify critical transcription factors that are regulated by ECM, and to use EGFR gene as a model. Since EGFR is not regulated by ECM at transcription level, we performed most of the proposed experiments using mammary-specific casein genes as models. We identified that transcription factors Stat5 and C/EBP $\beta$  were regulated by ECM. Results showed that ECM cooperates with prolactin to induce Stat5 phosphorylation and nuclear translocation in Eph4 cells. Chromatin immunoprecipitation (ChIP) assays demonstrated that levels of acetylated histones as well as binding of Stat5 and C/EBP $\beta$  in the casein promoters increased in response to ECM and prolactin treatment. These results directly addressed the central questions we asked in the proposal. Above and beyond that, we showed that trichostatin A (TSA)-induced histone acetylation failed to activate  $\beta$ -casein expression, suggesting that histone acetylation is not sufficient for the gene transcription. Introduction of a dominant negative Brg1, an ATPase subunit of SWI/SNF complex, significantly reduced both  $\beta$ - and  $\gamma$ -casein expression, indicating that SWI/SNF-dependent chromatin remodeling is required for transcription of these two milk protein genes. Thus, ECM-regulated transcription of the mammary-specific casein genes requires the concerted action of histone acetylation, ATP-dependent chromatin remodeling, and activation of transcription factors.

## **Body:**

### **Part I: Global histone acetylation in 3D lrECM does not depend on the formation of polarized acini structures.**

Culturing non-malignant mammary epithelial S1 cells in 3D within laminin-rich ECM (lrECM) allows the cells to form a polarized 3D structure (tissue-like acini) reminiscent of mammary alveolar structures *in vivo* (Fig. 1A) whereas this morphological differentiation does not occur when S1 cells are cultured as 2D monolayers. To address the question in specific aim I, we first detect the global histone acetylation in S1 cells cultured in 2D and 3D. The tissue-like morphogenesis observed in 3D is accompanied by a 60% decrease of acetylated H4 levels (Fig. 1B). A decrease of histone H3 acetylation is also detected in this condition (Fig. 1B). To determine whether the global histone deacetylation is a result of the acinar morphogenesis, we measured the level of acetylated H3 and H4 in T4 cells, the tumorigenic derivative of the S1 cells. As expected, T4 cells cultured in 3D within lrECM formed large non polarized and disorganized colonies reminiscent of tumors (Fig. 1C) (1). Nevertheless T4 cells in 3D lrECM are round and form clusters unlike the flatten cells on tissue culture plastic (Fig. 1C), and levels of acetylated H4 and H3 were decreased in 3D culture (Fig. 1D). Both S1 and T4 cells spread out in 2D, and exhibited prominent stress fibers as well as cortical actin when stained with fluorescently-tagged phalloidin (Fig. 1E). Even though the gross architectures of the colonies formed in 3D by these cells differed, individual S1 and T4 cells were similarly rounded in 3D (Fig. 1A, C ) with mainly cortical actin (Fig. 1E). Altogether these results suggest that the organization in well-polarized acini does not correlate with a global histone deacetylation.

### **Part II: Histone deacetylation in 3D culture is correlated with global reduction in gene expression.**

We proposed that change in chromatin organization during normal breast specialization could control specific gene expression. One question arising from **Part I** is whether this substantial histone deacetylation in 3D culture regulates gene expression. Indeed, broad acetylation of histones H3 and H4 has been shown to lead to chromatin decondensation and a structure permissible for transcription [reviewed in (5)] whereas histone deacetylation correlates with the repressed state of the chromatin structure and alterations in patterns of gene expression (6). To determine whether culture in 3D lrECM induced a reduction in gene expression in addition to global histone deacetylation, we collaborated with Aylin Rizki to perform microarray analysis. We found that cells cultured in 3D lrECM showed a reduction in gene expression as compared to cells cultured in 2D (Fig. 2A). Of the differentially expressed genes, 162 genes were expressed at lower levels in 3D, i.e. exhibiting a mean ratio of 3D/2D below 1, compared to only 91 expressed at higher levels i.e. exhibiting a mean ratio of 3D/2D up to 1. This decrease in gene expression is consistent with the global histone deacetylation observed in 3D culture. These results addressed the question in specific aim I at the whole genome level.

To determine whether the lrECM-dependent global deacetylation could specifically affect a functionally relevant gene, we proposed in specific aim 1 to determine whether ECM-mediated EGFR down regulation corresponds with histone deacetylation in the promoter of this gene. This hypothesis was based on the fact that protein levels of EGFR are downregulated in 3D, previous reports which had shown EGFR mRNA levels in different breast cancer cell lines correlate with the amount of receptor protein (7) and our initial findings by RT-PCR that the mRNA appeared to be regulated as well. We have now employed a more sensitive and quantitative method for measuring the mRNA level of EGFR, utilizing realtime PCR technology and find no significant difference between cells cultured on tissue culture plastic and cultured in 3D lrECM (Fig 2B). The reasons of the discrepancy between our data and previous data may be a more sensitive method of detection, the fact that they studied the EGFR expression in tumor cell lines verses a non-malignant cell line or that the difference in EGFR protein levels between 2D and 3D culture is controlled at a post-transcription level.

To address the question we raised in specific aim I, we detected transcription of p21 gene in S1 cells cultured in 2D and 3D. p21(WAF1) is a member of CIP/KIP family which inhibit CDKs activity. It plays an important role in p53 mediated cell cycle arrest induced by DNA damage (8). From our array data, the level of p21 in S1 cells in 3D was reduced to 76% of that in cells in 2D (data not shown) and it had previously been shown that the expression of the p21 gene is controlled by the acetylation status of the histones associated to its promoter (9) (10). Quantitative real-time RT/PCR confirmed the array data that p21 mRNA levels were reduced in 3D as compared to 2D (Fig. 2C). To determine whether this reduction in expression is correlated with reduced acetylation at the promoter region of the p21 gene, we performed chromatin immunoprecipitation (ChIP) experiments. Lysates from S1 cells cultured in 2D or 3D were immunoprecipitated for acetylated histones H3 or H4, and the remaining associated DNA was PCR-amplified using primers specific for the p21 promoter. Culturing the cells in 3D lrECM induces a dramatic reduction of histone H4 acetylation associated with the p21 promoter as compared to 2D cultures (Fig. 2D). This result is consistent with the p21 reduced expression observed by microarray analysis and RT/PCR. All these data indicate that lrECM-induced histone deacetylation leads to functional reduction of gene expression.

### **Part III: Transcription factor Stat5 and C/EBP $\beta$ are regulated by ECM to induce transcription of mammary-specific gene**

In specific aim II, we proposed to identify the ECM-regulated transcription factors during the differentiation of mammary epithelial cells, and to use EGFR gene as a model. We showed in Part I and II that the differentiation of S1 cells in 3D lrBM is accompanied by rearrangements of the chromatin structure. However, EGFR gene may not be a good model to study the transcriptional regulation during the differentiation of mammary gland epithelial cells induced by extracellular matrix (ECM); because this gene appears to be regulated posttranscriptionally in 3D lrECM. In addition, the formation of acini-like structures in S1 cells does not achieve the fully differentiated phenotype in that they do not express milk proteins. A wealth of studies have shown that tissue specific gene transcription is regulated in discrete and controlled stages, from the changing of

chromatin structure to the recruitment of protein complexes to the promoter and enhancer (11). We therefore, have chosen to use mammary specific genes as models, and investigated ECM-regulated transcription in mammary epithelial cell line EpH4, which forms acini-like structure in lrECM but also can fully differentiate to express milk proteins.

The gene encoding the milk protein,  $\beta$ -casein, has been used widely as a marker for functional differentiation of MECs. We and others have shown that in both primary mouse mammary epithelial cells and immortalized mammary epithelial cell lines (12-14), transcription of  $\beta$ -casein requires signals from both laminin-111 (previously named laminin-1) and prolactin (15-20). Using EpH4, an epithelial cell line derived from normal mouse mammary gland (12, 13), we observed that  $\beta$ - and  $\gamma$ -casein mRNA levels were highly upregulated in response to prolactin and lrECM treatment (Fig. 3A), which correlated with significant changes in cellular morphology (data not shown). Furthermore, consistent with previous studies (2, 19), we established that laminin-111 indeed is the lrECM constituent that induces  $\beta$ -casein expression in EpH4 cells (data not shown).

As we proposed in specific aim I, we amplified and cloned the promoter region from -340 to -1 into a luciferase reporter vector and stably transfected the reporter plasmid into EpH4 cells. Luciferase activity was dramatically induced in EpH4 cells after treatment with lrECM and prolactin (Fig. 3B), indicating that the  $\beta$ -casein promoter is transcriptionally activated in these cells. Consistent with the PCR results, neither prolactin nor lrECM alone could appreciably enhance promoter activity. These results indicate that the ECM regulates  $\beta$ -casein transcription through activation of the promoter.

A number of transcription factors, including STAT5, C/EBP $\beta$ , and GR, have been shown to be involved in transcription of mammary specific genes [reviewed in (15)]. STAT5 and C/EBP $\beta$  binding sites were also identified in the bovine  $\beta$ -casein ECM-response element, BCE-1 (21). To determine whether these two factors regulate ECM- and prolactin- induced expression of the endogenous  $\beta$ -casein gene, we have detected expression and localization of Stat5 and C/EBP $\beta$  in Eph4 cells after treatment with ECM and prolactin. Although the total level of STAT5 did not change, the levels of phosphorylated STAT5 and its nuclear translocation increased after combined treatment with lrECM and prolactin. However, neither treatment alone could induce these changes (Fig. 3C, 3D). Total cell and nuclear levels of C/EBP $\beta$  remained unchanged after the treatments (Fig. 3C, 3D).

To determine whether STAT5 and C/EBP $\beta$  become associated with the  $\beta$ -casein promoter after ECM and prolactin treatment, we performed ChIP assays. Addition of these two compounds significantly increased the association of STAT5 and C/EBP $\beta$  with the  $\beta$ -casein promoter, whereas the interaction between the promoter and GR remained at the control level (Fig. 4A). The promoter of the  $\beta$ -amylase gene, which is not expressed in mammary epithelial cells, was included as a negative control and was not detected in any of the ChIP samples (data not shown). Thus, exposure of EpH4 cells to lrECM and prolactin increases both STAT5 levels in the nucleus, and the binding of this factor and C/EBP $\beta$  to the  $\beta$ -casein promoter. We also found that treatment with prolactin and lrECM moderately enhanced the binding of Brg1, the ATPase subunit of SWI/SNF complex, to the  $\beta$ -casein promoter. Analysis of the DNA immunoprecipitated with a RNA polymerase

II antibody showed an increased association of this protein with the  $\beta$ -casein promoter in response to lrECM and prolactin (Fig 4A). These results indicate that extracellular matrix and prolactin regulate DNA binding activity of stat5 and C/EBP $\beta$  to induce mammary-specific gene transcription, and directly address the central question we asked in specific aim II.

Binding sites of STAT5 and C/EBP $\beta$  were identified in promoters of other milk protein genes, such as  $\gamma$ -casein (22). We asked whether other milk protein genes are regulated similarly as  $\beta$ -casein. The association of these factors with the  $\gamma$ -casein promoter was determined by ChIP assays. We found that treatment with prolactin and lrECM enhanced binding of STAT5 and C/EBP $\beta$ , and increased Brg1 and RNA Polymerase II levels in the promoter region of  $\gamma$ -casein gene (Fig. 4B). The mouse casein genes cluster at a single gene locus on chromosome 5 in this order:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\kappa$  (23), and the expression of casein genes is coordinately regulated during pregnancy and lactation (24). Thus, the binding of these transcription factors and the chromatin remodeling complex together appears to activate the entire gene locus.

We showed above that treatment with lrECM and prolactin induced the recruitment of transcription factors and the SWI/SNF complex to the  $\beta$ -casein promoter. To determine whether ECM and prolactin control these events separately or cooperatively, we performed ChIP analysis after cells were treated either singly or with both agents. We found that STAT5 bound to the  $\beta$ -casein promoter in cells treated with both lrECM and prolactin, but treatment with either component alone failed to induce appreciable binding (Fig. 4C). These results are consistent with the western blot data showing that nuclear translocation of STAT5 depends on both the ECM and hormonal signals (Fig. 3D). Combined lrECM and prolactin treatment also induced binding of C/EBP $\beta$  to the  $\beta$ -casein promoter (Fig. 4C). Recruitment of Brg1 and RNA polymerase II in the  $\beta$ -casein promoter required both lrECM and prolactin as well (Fig. 4C). These results establish that ECM cooperates with prolactin to induce the binding of transcription factors as well as the transcriptional machinery to the  $\beta$ -casein promoter.

#### **Part IV: Histone acetylation is involved in but not sufficient to the transcriptional activation of $\beta$ -casein gene**

Previously, we showed that treatment with histone deacetylase inhibitors could partially substitute for lrECM in activating a stably integrated bovine ECM-response element (BCE-1) in a mammary epithelial cell line (CID-9), suggesting that histone acetylation may play a role in transcriptional regulation of this enhancer (21). Surprisingly, however, the same treatment was later shown to inhibit transcriptional activation of the endogenous  $\beta$ -casein gene (25). Here we sought to determine whether histone acetylation is involved in transcriptional regulation of the endogenous  $\beta$ -casein gene. ChIP assays using antibodies against acetylated histone H3 and H4 demonstrated enhanced histone acetylation in the  $\beta$ -casein promoter, but not the  $\beta$ -amylase promoter, in response to treatment with lrECM and prolactin (Fig 5A). In addition, neither lrECM nor prolactin alone induced histone acetylation in the  $\beta$ -casein promoter (data not shown), confirming that the cooperation between the two signals is important.

To determine whether the increase of acetylated histone in the  $\beta$ -casein promoter was sufficient to induce transcription of the endogenous gene, EpH4 cells were treated with TSA in the presence or absence of ECM and prolactin. ChIP data showed that the levels of acetylated histone H4 (AcH4) appreciably increased in the  $\beta$ -casein promoter (Fig. 8B). Quantitative PCR showed, however, that the level of  $\beta$ -casein mRNA was increased by only 1.6 fold in undifferentiated cells after TSA treatment; the levels of both total and phosphorylated STAT5, C/EBP $\beta$ , and GR did not change (Fig. 5C, 5D). In the functionally differentiated cells that were cultured with prolactin and IrECM, TSA treatment significantly suppressed the induction of  $\beta$ -casein expression. Western blot analysis showed that phosphorylated STAT5 levels decreased in TSA treated cells, suggesting that this inhibition may be due to an indirect effect of TSA on STAT5 phosphorylation (Fig. 5C, 5D). These results now clarify previous contradictions and indicate that histone acetylation alone is not sufficient to induce transcription of the endogenous  $\beta$ -casein gene above the basal level.

#### **Part V: ATPase activity of SWI/SNF is critical for $\beta$ -casein transcription.**

A point mutation in the ATP-binding site of Brg1 was shown to abolish its ATPase activity, and produce a dominant negative effect on chromatin remodeling function of the SWI/SNF complex (26). To examine whether this ATPase was the additional factor required for  $\beta$ -casein expression, we generated a mammary cell line that conditionally expressed Flag-tagged DN-Brg1 under the control of a tetracycline-repressible transactivator. Withdrawal of tetracycline from culture medium for 2 days caused a dramatic induction of DN-Brg1 expression (Fig. 6A) and a significant repression in  $\beta$ - and  $\gamma$ -casein transcription (Fig. 6B), while lactoferrin and GAPDH transcription remained unaltered. Thus, the ATPase activity of SWI/SNF is necessary for transcriptional activation of casein genes. The binding of STAT5 and C/EBP $\beta$ , as well as the levels of AcH4 in the  $\beta$ -casein promoter in ECM- and prolactin- treated cells did not change significantly in response to DN-Brg1 expression (Fig. 6D). Western blot analysis revealed that DN-Brg1 expression did not affect the nuclear levels of STAT5, C/EBP $\beta$  and GR as well (data not shown). These results rule out the possibility that expression of DN-Brg1 inhibited  $\beta$ -casein transcription indirectly by repressing the activity of transcription factors, and suggest that transcription factor binding and histone acetylation in the  $\beta$ -casein promoter are events that take place upstream of Brg1 ATPase activity and are not dependent on SWI/SNF. In contrast, recruitment of RNA polymerase II to the  $\beta$ -casein promoter was inhibited by DN-Brg1 expression, suggesting that its function in mediating transcription of casein genes occurs downstream of SWI/SNF, and therefore, is dependent on ATP-dependent chromatin remodeling (Fig. 6D).

Formation of the SWI/SNF complex was shown to occur independently of its ATPase activity in NIH 3T3 cells (27, 28). We asked whether the SWI/SNF complex interacts with STAT5, C/EBP $\beta$  and GR, and whether the ATPase activity of Brg1 is necessary for this interaction in mammary epithelial cells. Protein complexes from control and DN-Brg1-expressing cells were immunoprecipitated with agarose beads conjugated with anti-FLAG M2 antibody. The immunoprecipitated protein complexes were analyzed by western blot using antibodies against STAT5, C/EBP $\beta$ , GR, and lamin B. STAT5 co-immunoprecipitated with the DN-Brg1 in the DN-Brg1-expressing cells, but it was absent

from the immunoprecipitate from the non-expressing cells (Fig. 7A). We also detected interactions between DN-Brg1 and GR, and DN-Brg1 and C/EBP $\beta$  in lysates from the DN-Brg1-expressing cells (Fig. 7A). However, lamin B did not associate with Brg1, suggesting that the interaction between STAT5, C/EBP $\beta$ , GR and the SWI/SNF complex is specific (Fig. 7A). The association of SWI/SNF with GR and C/EBP $\beta$  has been shown (29, 30), but the interaction between SWI/SNF and STAT5 has not been reported previously. To confirm the co-immunoprecipitation (co-IP) results in DN-Brg1-expressing cells, we performed a co-IP experiment using the parental cells. The results showed that endogenous wild type SWI/SNF was bound to STAT5 in lrECM- and prolactin-treated EpH4 cells but not in control cells (Fig. 7B). Therefore, the SWI/SNF chromatin remodeling complex may be recruited to the  $\beta$ -casein promoter by STAT5, C/EBP $\beta$ , and/or GR. Several other milk proteins, including  $\alpha$ -casein,  $\gamma$ -casein, whey acidic protein (WAP), and  $\beta$ -lactoglobulin have been shown to be regulated by ECM and lactogenic hormones (15, 31-33). The promoter or enhancer elements of these genes contain binding sites for STAT5 and GR (15, 22, 34). Interestingly, DN-Brg1 expression significantly inhibited  $\gamma$ -casein transcription, but had no detectable effect on transcription of the lactoferrin gene (Fig 6B). These data are consistent with the finding that the expression of lactoferrin is not dependent on the cooperation of ECM and prolactin signals in MECs, and that basal transcriptional regulation may be different for lactoferrin expression (35). These results indicate that transcription factors such as STAT5 by binding to specific promoters determine recruitment of the SWI/SNF complex to allow expression of milk protein genes.

### **Key research accomplishments:**

1. Histone deacetylation in 3D culture is correlated with global reduction in gene expression.
2. ECM-regulated p21 down-regulation is associated with histone deacetylation in the promoter region.
3. Activation of Stat5 and C/EBP $\beta$  was regulated by ECM and prolactin to induce mammary-specific gene expression.
4. Histone acetylation contributes to, but is not sufficient to induce mammary-specific gene expression.
5. ATPase dependent chromatin remodeling is required for the transcriptional activation of mammary specific gene.

### **Reportable Outcomes:**

- 1, Manuscript, title: Extracellular Matrix, Nuclear and Chromatin Structure and Gene Expression in Normal Tissues and Malignant tumors: A Work in Progress. Virginia A. Spencer., **Ren Xu.**, Mina J. Bissell., *Advances in Cancer Research*, in press.

2, Presentation at Asilomar Chromatin and Chromosomes Conference 2005; Title: SWI/SNF cooperates with Stat5 and C/EBP $\beta$  to regulate functional differentiation in MECs.

3, Abstract published at ASCB 2005 Annual Conference, Title: SWI/SNF complex and histone acetylation cooperate with Stat5 and C/EBP $\beta$  to regulate  $\beta$ -casein transcription

4, Abstract published at Era of Hope 2005 - Department of Defense Breast Cancer Research Program Meeting, Title: Chromatin Remodeling Cooperates with Transcription Factors to Regulate Functional Differentiation of Mammary Epithelial Cells

5, Abstract published at AACR chromatin, chromosomes, and cancer epigenetics meeting 2004, Title: From extracellular signaling to chromatin remodeling: how do extracellular matrix and prolactin regulate  $\beta$ -casein transcription.

6, Abstract published at ASCB 2003 Annual Conference, Title: SAHA treatment phenotypically reverts the malignant HMT3522 T4-2 human breast cell line in three dimension culture.

## **Conclusion:**

We demonstrate that ECM induces global histone deacetylation in human breast epithelial cells, and that this deacetylation correlated with a global reduction in gene expression. These results indicate a tight link between ECM-controlled cell function and chromatin structure, which plays important roles in the transcriptional regulation. Using  $\beta$ - and  $\gamma$ -casein genes as models, we identified STAT5 and C/EBP $\beta$  as two ECM-regulated transcription factors. The activation of  $\beta$ -casein gene is associated with histone acetylation in the promoter regions. We further show that chromatin remodeling induced by histone acetylation is not sufficient for assembly or stabilization of the RNA transcriptional machinery on the  $\beta$ -casein promoter, and that this process depends on ATP-dependent chromatin remodeling (Fig. 7C). Such precise regulation from extracellular signals to chromatin structure is most likely fundamental to mammary gland development and function to ensure control of milk protein gene expression during lactation, a process that is vital to the offspring's survival. Since the malignant mammary epithelial cells can not express milk proteins under differentiate condition, it will be interesting to detect whether the activation of transcription factors and chromatin remodeling are impaired in the malignant cells.

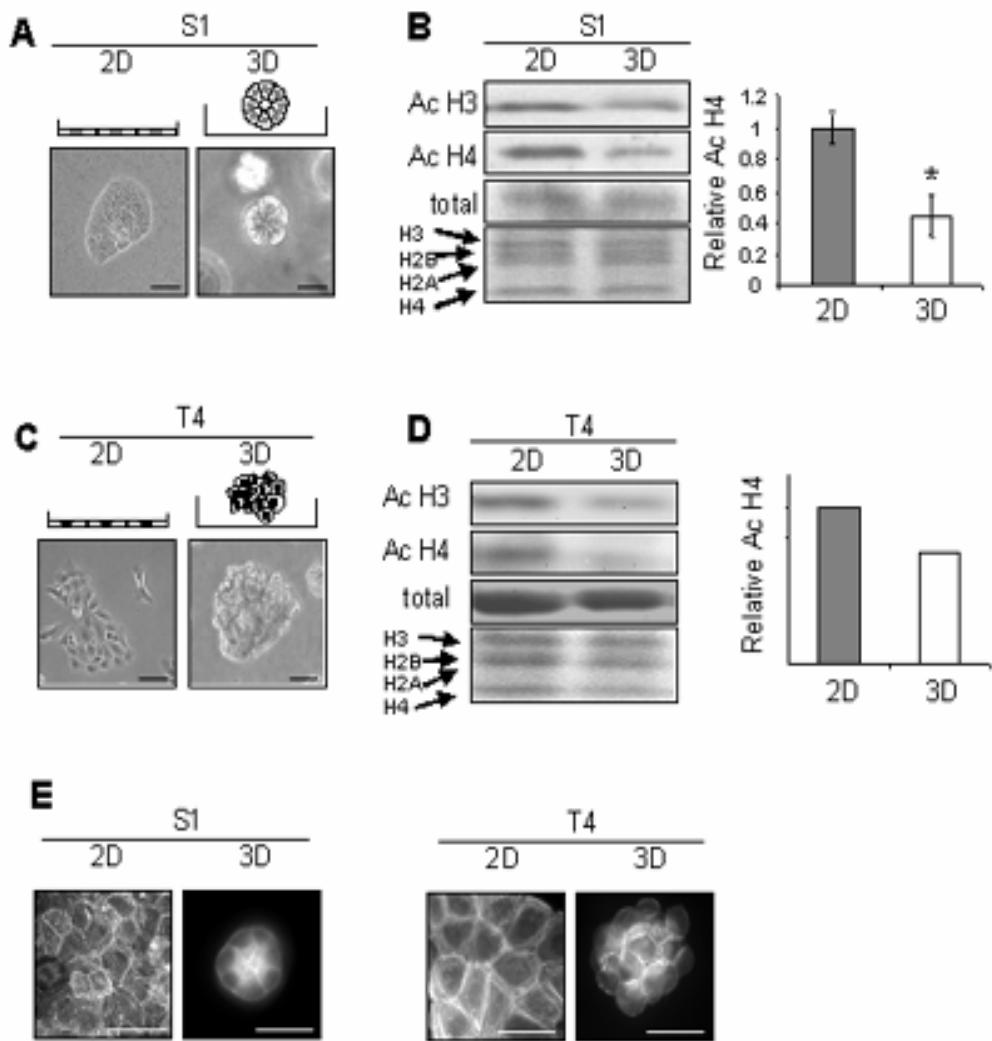
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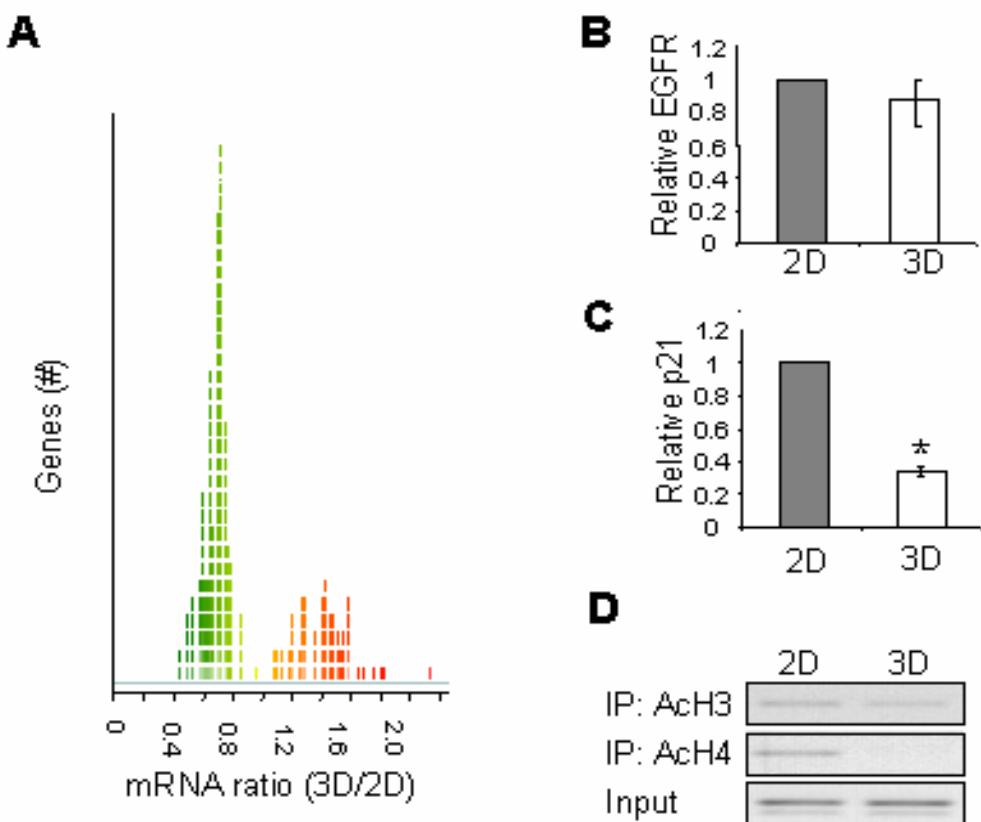
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Figure 1:



**Figure 1.** Culture in 3D lrECM induces alterations in cellular morphology and global histone deacetylation. **(A)** Phase contrast images of S1 non-malignant human mammary epithelial cells on tissue culture plastic (2D) or within lrECM (3D). **(B)** Western blot analysis of acetylated histones H3 (Ac H3) and H4 (Ac H4) in S1 cells cultured in 2D and 3D. Bar graph showing relative acetylated histone levels in S1 cells cultured in 2D and 3D. Error bars indicate s.e.m; (\*) p<0.01; **(C)** Phase contrast images of T4 malignant human mammary epithelial cells on tissue culture plastic (2D) or within lrECM (3D). **(D)** Western blot analysis of acetylated histones H3 (Ac H3) and H4 (Ac H4) in T1 cells cultured in 2D and 3D. Bar graph showing relative acetylated histone levels in T4 cells cultured in 2D and 3D. Error bars indicate s.e.m; (\*) p<0.01; **(E)** Immunofluorescence images of phalloidin-stained S1 and T4 cells cultured in 2D or 3D. scale bars, 50  $\mu$ m.

Figure 2:



**Figure 2.** Culture in 3D lrECM induces a global reduction in gene expression. **(A)** Ratio of global mRNA levels for S1 cells cultured in 2D or 3D lrECM. The x-axis shows mean ratio of 3D/2D for four experiments; all genes with  $p < 0.01$  are displayed: 91 genes have higher mRNA levels in 3D; 162 genes have lower levels in 3D. **(B, C)** Quantitative RT/PCR analysis for EGFR (B) and p21 (C) mRNA levels, normalized to levels of 18S in the same samples. **(D)** ChIP assay measuring Ac H3 and Ac H4 levels associated with the p21 promoter region for S1 cells cultured in 2D or 3D lrECM.

Figure 3:

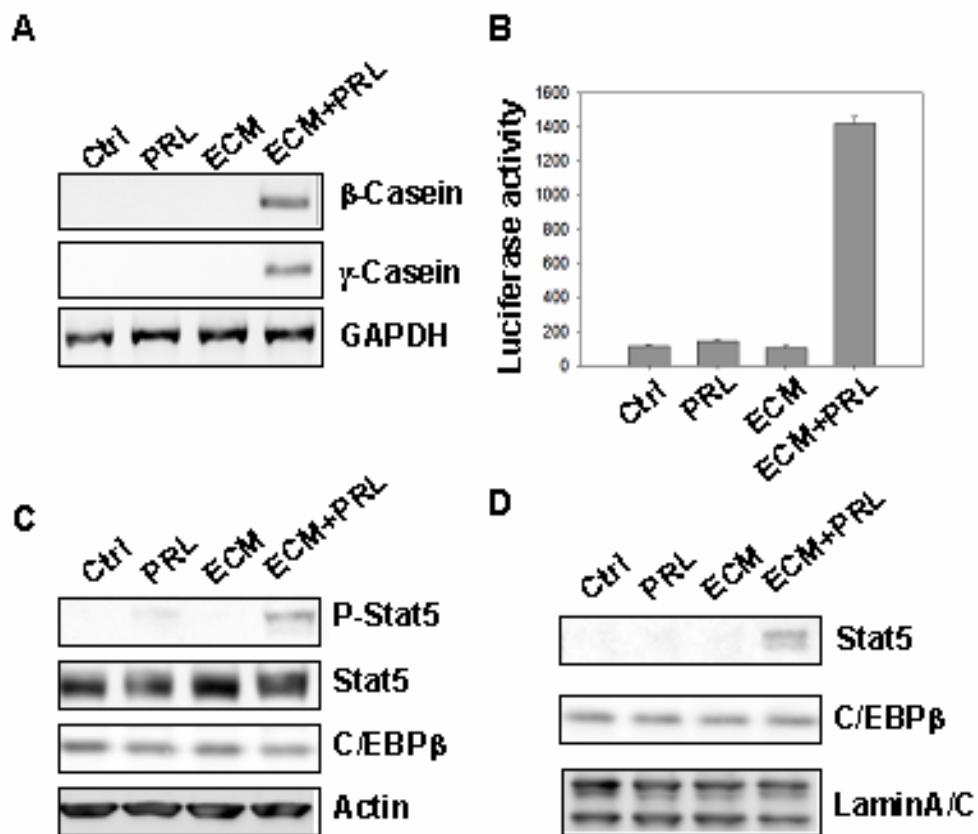


Figure 3: Stat5 phosphorylation and nuclear translocation are activated in EpH4 cells in response to ECM and prolactin treatment. (A)  $\beta$ - and  $\gamma$ -casein expression was determined by RT-PCR in EpH4 cells cultured for 2 days in GIH (DMEM/F12 supplemented with 5  $\mu$ g/ml insulin and 1  $\mu$ g/ml hydrocortisone) media alone (Ctrl), GIH + 3  $\mu$ g/ $\mu$ l prolactin (PRL), GIH + 2% lrECM (ECM), and GIH + 3  $\mu$ g/ $\mu$ l prolactin + 2% lrECM (ECM+PRL). GAPDH cDNA was used as a loading control. (B) The  $\beta$ -casein promoter was cloned into pGL3 luciferase vector, and stably transfected into EpH4 cells. The  $\beta$ -casein promoter activity was determined by luciferase assays. (C, D) Transcription factor levels in the cell lysates (C) and nuclear lysates (D) of EpH4 cells were determined by western blotting.

Figure 4:

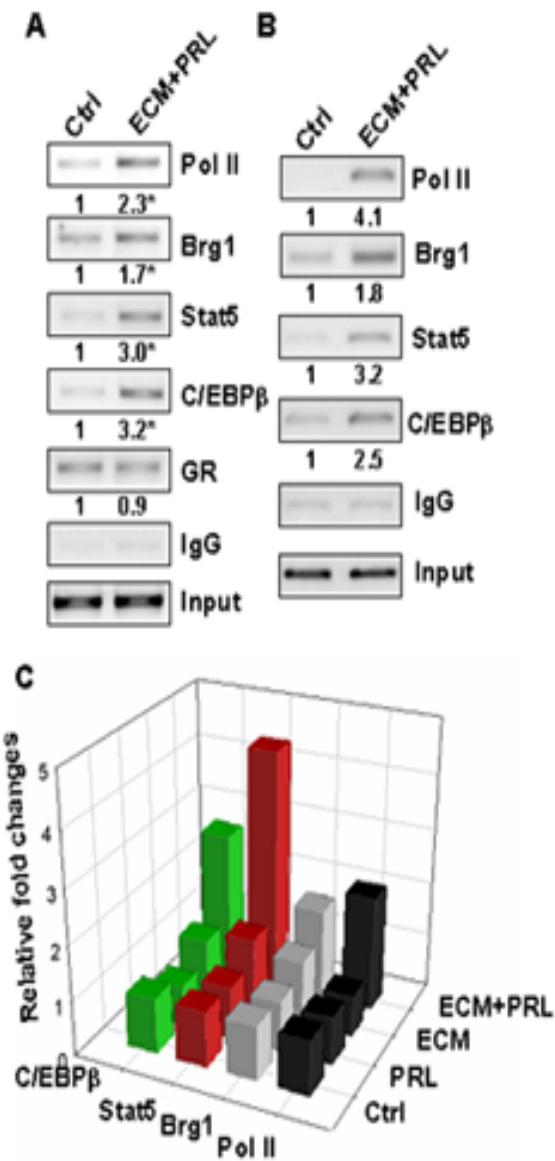


Figure 4: Binding of transcription factors and the SWI/SNF complex to the  $\beta$ -casein promoter is regulated by ECM and prolactin. (A, B) ChIP assays followed by PCR analysis to detect the binding of STAT5, C/EBP $\beta$ , GR, Brg1, and RNA Polymerase II in the  $\beta$ -casein (A; n=4) and  $\gamma$ -casein (B; n=2) promoters. The PCR results were quantified by AlphaEaseFC software, and the values of bound DNA were normalized to input DNA. Fold enrichments were determined by dividing the normalized values from treated cells by that of untreated cells, \* p<0.05. (C) Quantification of ChIP results in EpH4 cells treated with prolactin (PRL), lrECM (ECM), or prolactin plus lrECM (PRL+ECM). Graph displays the mean of two experiments.

Figure 5:

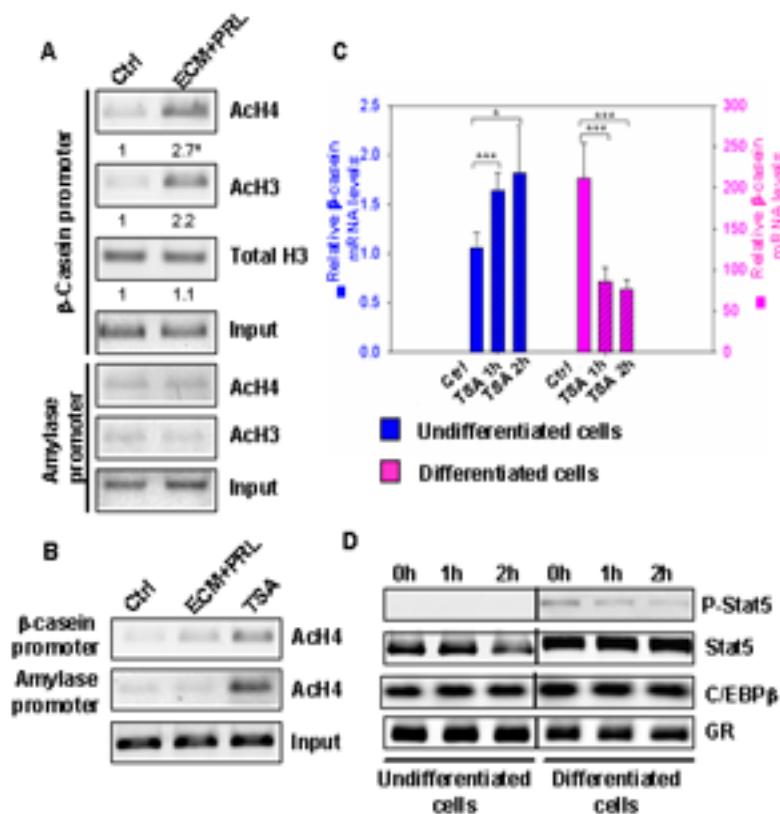


Figure 5: Histone acetylation contributes to, but is not sufficient to induce  $\beta$ -casein expression. (A) The levels of total histone H3, as well as acetylated histone H4 and H3 in the  $\beta$ -casein promoter were measured by ChIP analysis. The  $\beta$ -Amylase promoter was used as a control, \* p<0.05 (n=3). (B) The levels of AcH4 in the  $\beta$ -casein promoter were determined by ChIP assays in control and TSA (80 nM) treated cells. (C) The  $\beta$ -casein mRNA levels in undifferentiated EpH4 cells (in GIH medium) and differentiated cells (in GIH plus prolactin and lrECM) were measured by quantitative RT-PCR after TSA treatment. Graph displays average  $\pm$  SEM; \* p<0.05, \*\*\* p<0.01 (n=4). (D) Protein levels of phosphorylated STAT5, total cell STAT5, C/EBP $\beta$  and GR were analyzed by western blotting after TSA treatment.

Figure 6:

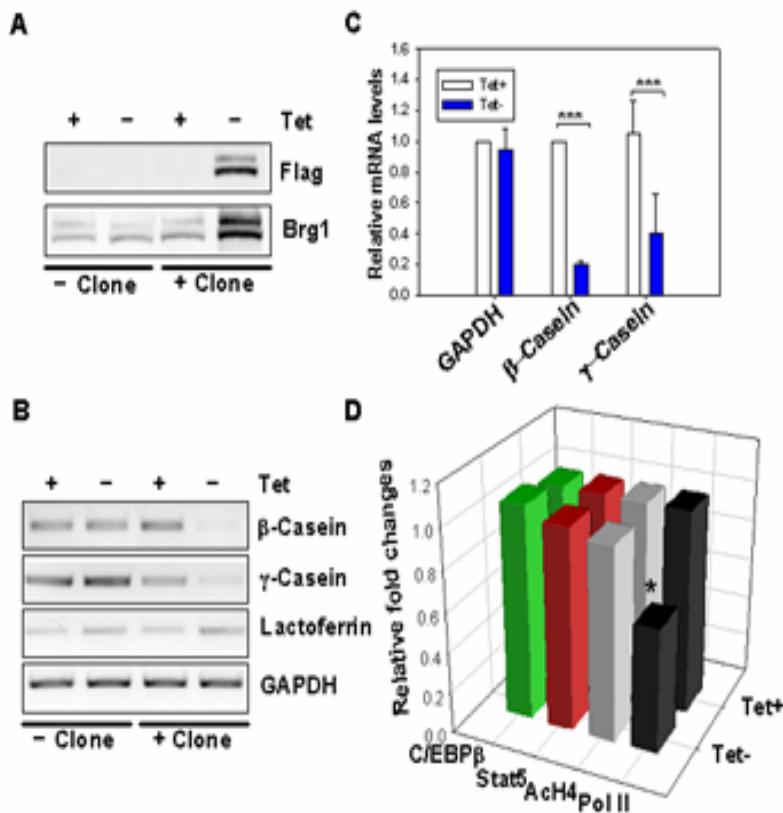


Figure 6: DN-Brg1 expression in EpH4 cells suppresses transcription of the  $\beta$ -casein gene. (A) Western blot analysis of DN-Brg1 expression in stably transfected EpH4 cells. (B, C) RT-PCR (B) and quantitative PCR (C) analysis of the levels of  $\beta$ - and  $\gamma$ -casein genes in DN-Brg1-expressing and non-expressing cells. Graph displays average  $\pm$  SEM; \*\*\* p<0.01 (n=4). (D) ChIP assays showing the levels of AcH4 and the binding activity of STAT5 and C/EBP $\beta$  in the  $\beta$ -casein promoter in DN-Brg1-expressing cells. Graph displays mean of three experiments, \* p<0.05.

Figure 7:

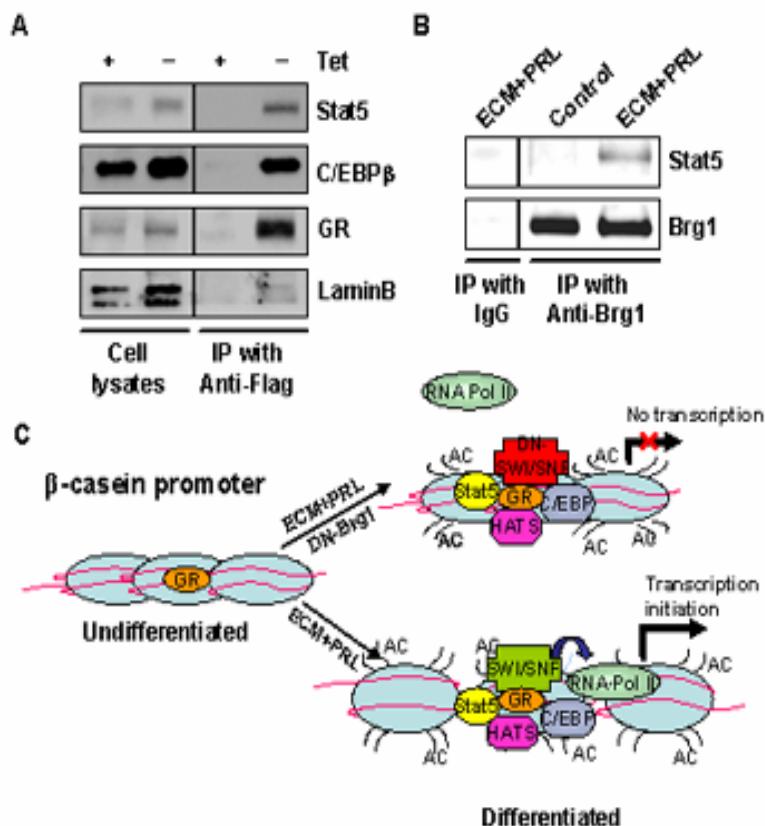


Figure 7: STAT5, C/EBP $\beta$ , and GR interact with DN-Brg1 in EpH4 cells. (A) Interaction between transcription factors and DN-Brg1 was determined by co-IP analysis. Total lysates before immunoprecipitation were used as input control. (B) Interaction between endogenous Brg1 and STAT5 was detected by co-IP analysis. (C) Model displaying how exposure of mammary epithelial cells to ECM and prolactin induces the recruitment of transcription factors and chromatin remodeling enzymes to the  $\beta$ -casein promoter, and how aberrations in SWI/SNF function interfere with RNA polymerase II recruitment.